REMARKS

Claims 41, 42, 45, 46, 52-55, 57 and 82 are rejected on the ground of nonstatutory double patenting in view of claims 1-8, 19 and 20 of U.S. Pat. No. 6,767,719. Claims 43, 44, 47-51, 56, and 74-81 were objected to as depending from a rejected base claim.

Interview

Applicants thank the Examiner Angell for the courtesy of a telephonic interview on December 5, 2008. The substance of the discussion corresponds to the comments below. The Examiner agreed that the claims as amended would be allowable.

Amendment to the Title

The title is amended to more clearly describe the subject matter of the claims.

Claim Amendment

Claim 41 has been amended at the request of Examiner Angell to improve clarity. The amendment does not have any effect on the meaning or scope of the claim.

Obviousness-Type Double Patenting

Claims 41, 42, 45, 46, 52-55, 57 and 82 were rejected on the ground of nonstatutory double patenting in view of claims 1-8, 19 and 20 of US Pat. No. 6,767,719.

Applicants respectfully traverse the rejection. Initially it is noted that the claims of the '719 patent relate to polynucleotides encoding mouse telomerase reverse transcriptase protein (mTRT) while the claims of the instant invention relate to polynucleotides encoding human telomerase reverse transcriptase protein (hTRT). Both claim sets refer to polynucleotides encoding a polypeptide with "SEQ ID NO:2" but the sequences of the two specifications are not the same. *See* Greenberg et al., 1998, "Expression of mouse telomerase reverse transcriptase during development, differentiation and proliferation" *Oncogene* 16:1723-1730 which states the overall identity between the mouse and human TRT sequences is about 64% (page 1724, column 1, bottom; see Appendix A). Also enclosed for the convenience of the Examiner is an alignment

of the human TRT sequence (from the instant application) and the mouse TRT sequence (from the '719 patent) showing overall identity of about 62% (Appendix B).

Although the Office discusses the claims of the current application and the '719 patent as being broadly similar (e.g., each encompassing a polypeptide with a "T motif" that increases proliferative capacity of a cell) a proper double patenting analysis requires showing that the claimed inventions are obvious variants of each other. The Office also states that the claims of the '719 patent are drawn to a polynucleotide that "is encompassed" by the instantly claimed polynucleotide. Applicants respectfully submit that is not correct and that the double patenting rejections should be withdrawn.

The instant application has one independent claim (claim 41), as shown below. The claims of the '719 patent cited by the Office (claims 1-8, 19 and 20) include three independent claims (claims 1, 2, and 3), as shown below.

> Claim 41 if the instant application requires, *inter alia*, a polynucleotide that encodes a protein comprising SEQ. ID NO:2 [**human TRT**], or fragment of SEQ. ID NO:2 that contains the telomerase T motif: Trp-X_{12}-Phe-Phe-Tyr-X-Thr-Glu-X_{10-11}-Arg-X_3-Trp-X_7-Ile.
>
> Claim 1 of the '719 patent requires, *inter alia*, that the encoded telomerase reverse transcriptase protein "has at least 90% sequence identity to SEQ. ID NO:2 [**mouse TRT**] . . ."
>
> Claim 2 of the '719 patent requires, *inter alia*, a polynucleotide that encodes a telomerase reverse transcriptase having the amino acid sequence of SEQ. ID NO:2 [**mouse TRT**]"
>
> Claim 3 of the '719 patent requires, *inter alia*, a polynucleotide comprising SEQ. ID NO:1 [**mouse TRT**], or fragment thereof that encodes a protein having telomerase activity . . . "

Applicants respectfully submit the invention of Claim 41 of the instant application is not an obvious variant of an invention claimed in the '719 patent.

Claim 1 of the '719 patent (the first independent claim) is directed to a polynucleotide encoding a polypeptide that "has at least 90% sequence identity to SEQ. ID NO:2 [mouse TRT] . . ." Claim 41 of the instant application refers to a polynucleotide encoding a protein comprising SEQ ID NO:2 [human TRT]. SEQ ID NO:2 [human TRT] comprises less than 65% overall identity to the mouse TRT. Thus the polynucleotide of Claim 41 is neither encompassed by Claim 1 of the '719 patent nor an obvious variant of the polynucleotide of Claim 1 of the '719 patent. Moreover, although there are very short stretches of the human TRT sequence that have greater than 65% sequence identity with the corresponding region of the mouse TRT sequence, no fragment of human TRT encompassed by Claim 41 has at least 90% sequence identity to the mouse sequence. Accordingly the instantly claimed polynucleotide is not an obvious variant of Claim 1 of the '719 patent or any claim depending from Claim 1 of the '719 patent.

Claim 2 of the '719 patent (the second independent claim) is directed to a subgenus of the polynucleotides of SEQ ID NO:1 of the '719 patent. As is discussed above, and as the Office can easily discern from the record, the mouse and human TRT amino acid sequences are quite different and are encoded by different polynucleotides. The human TRT protein and nucleic acids encoding the protein are not obvious variants of the entirely different mouse TRT protein and polynucleotides.

Claim 3 of the '719 patent (the third independent claim) is directed to a polynucleotide comprising SEQ ID NO:1 (the mouse nucleic acid sequence) or a fragment thereof that encodes a catalytically active TRT containing at least one listed motif. As the Office can easily discern from the record, the mouse TRT DNA sequence (set forth as SEQ ID NO:1 of the '719 patent) and the human TRT DNA sequence (set forth as SEQ ID NO:1 of the instant application) are very different from each other. The instantly claimed polynucleotide is not an obvious variant of Claim 3 of the '719 patent or any claim depending from Claim 3.

Moreover, while Claim 41 is directed to a polynucleotide that encodes a TRT fragment containing the telomerase T motif Trp-X_{12}-Phe-Phe-Tyr-X-Thr-Glu-X_{10-11}-Arg-X_3-Trp-X_7-**Ile,** this motif is not found in the mouse TRT sequence (note the terminal isoleucine residue in the

motif which is a valine in the mouse sequence). For this reason as well, Claim 41 is not taught or suggested (or an obvious variant of) the claims of the '719 patent.

Thus, the invention of Claim 41 of the instant application is not an obvious variant of any invention claimed in the '719 patent. Conversely, inventions claimed in the '719 patent are not obvious variants of Claim 41 of the instant application.

Claim 41 of the instant application requires *inter alia*, a polynucleotide that encodes a protein comprising SEQ. ID NO:2 [**human TRT**], or fragment of SEQ. ID NO:2 that contains the telomerase T motif defined as Trp-X_{12}-Phe-Phe-Tyr-X-Thr-Glu-X_{10-11}-Arg-X_3-Trp-X_7-Ile. As noted above, there is little sequence identity between human and mouse TRT polypeptide sequences or the polynucleotide sequences encoding them. The polynucleotide claims of the '719 patent are directed to (1) a polynucleotide encoding mouse TRT (having a very different amino acid sequence than the human TRT); (2) a polynucleotide encoding a protein identical to or with at least 90% amino acid sequence identity to mouse TRT; and (3) a polynucleotide comprising SEQ. ID NO:1 [**mouse TRT**]. The mouse derived sequences are not obvious variants of the polynucleotide of Claim 41, which is a polynucleotide encoding a **human** TRT polypeptide, or a fragment containing a telomerase T motif with a terminal isoleucine.

For these reasons Applicants submit the obviousness-type double patenting rejections should be withdrawn.

Information Disclosure Statements

Applicants respectfully reiterate previous requests that the Examiner return initialed copies of IDSs filed by Applicants, including IDSs filed June 24, 2008, June 9, 2008, March 21, 2008, March 4, 2008, February 15, 2008, July 25, 2007 and February 23, 2003.,

Conclusion

Issuance of a Notice of Allowance is respectfully requested. If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 650-462-5330.

Respectfully submitted,

Randolph Ted Apple
Registration No. 36,429

Encls:
Appendix A: Greenberg et al.
Appendix B: Alignment

TOWNSEND and TOWNSEND and CREW LLP
Two Embarcadero Center, Eighth Floor
San Francisco, CA 94111-3834
Tel: 650-326-2400
Fax: 650-326-2422

61744444 v1

Oncogene (1998) 16, 1723–1730

http://www.stockton-press.co.uk/onc

Expression of mouse telomerase reverse transcriptase during development, differentiation and proliferation

Roger A Greenberg[1], Richard C Allsopp[2,4], Lynda Chin[1,3], Gregg B Morin[2] and Ronald A DePinho[1]

[1]Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, New York, 10461; [2]Geron Corporation, 230 Constitution Drive, Menlo Park, California 94025; [3]Division of Dermatology, Albert Einstein College of Medicine, Bronx, New York, 10461, USA

We have identified the mouse telomerase reverse transcriptase component (mTERT) and demonstrate both substantial sequence homology to the human ortholog (hTERT), and the presence of reverse transcriptase and telomerase specific motifs. Furthermore, we show functional interchangeability with hTERT in *in vitro* telomerase reconstitution experiments, as mTERT produces strong telomerase activity in combination with the human telomerase RNA component hTR. The mouse TERT is widely expressed at low levels in adult tissues, with greatest abundance during embryogenesis and in adult thymus and intestine. The mTERT component mRNA levels were regulated during both differentiation and proliferation, while mTR levels remained constant throughout both processes. Comparison of mTERT and mTR levels to telomerase activity indicates that mTERT expression is more tightly linked to the regulation of telomerase activity during these processes than is mTR. In contrast to the situation in human cell cultures, mTERT transcript levels are present at readily detectable levels in primary cultured cells and are not upregulated following crisis. The widespread expression of mTERT in primary cells and mouse tissues could explain the increased frequency of spontaneous immortalization of mouse cells in culture and tumorigenesis *in vivo*.

Keywords: telomerase; reverse transcriptase; mouse; telemere; cancer

Introduction

Telomeres are nucleoprotein complexes that cap the physical termini of all eukaryotic chromosomes (Greider, 1996) and play essential roles in the maintenance of chromosomal integrity and cell viability. Telomeres consist of lengthy G-rich simple repeat sequences that are synthesized *de novo* by a specialized reverse transcriptase known as telomerase (Greider and Blackburn, 1985; Yu *et al.*, 1990; Singer and Gottschling, 1994). The telomerase core enzyme consists of an RNA component and a polypeptide that bears classical structural motifs and functional activities of a reverse transcriptase (Lingner *et al.*, 1997; Nakamura *et al.*, 1997, 1998; Meyerson *et al.*, 1997; Harrington *et al.*, 1997; Kilian *et al.*, 1997; Weinrich *et al.*, 1997). As conventional DNA-dependent DNA polymerases fail to fully replicate the ends of chromosomes, telomerase has evolved to serve as the primary means by which eukaryotic cells maintain telomere length with each cell division cycle. Most somatic human tissues and primary cells possess low or undetectable telomerase activity, leading to a steady decline in telomere length with continued organ renewal *in vivo* and with passage in culture. This progressive telomere shortening has been shown to provide a signal for entry of cells into a state of replicative senescence (Harley *et al.*, 1990; Bodnar *et al.*, 1998; Kim *et al.*, 1994).

The frequent occurrence of high telomerase activity in immortalized cells and in cancers has led to the hypothesis that telomerase reactivation represents a critical step in the neoplastic process (Kim *et al.*, 1994). Indeed, the presence of moderate basal telomerase activity in some mouse somatic tissues as opposed to the lack of it in most humans tissues could provide a basis for the greater ease of immortalization and higher cancer incidence in the mouse (Prowse and Greider, 1995). The importance of telomerase reacivation in these settings may relate to the fact that marked genetic instability and cell death ensues as viral oncoproteins or mutational events drive cultures which would normally senesce, through additional rounds of replication coupled with accompanying telomere erosion (Counter *et al.*, 1992). However, the long telomeres and sustained growth of immortal human cultures lacking detectable telomerase activity and the ability of mouse embryonic fibroblasts (MEFs) nullizygous for the telomerase RNA to spontaneously immortalize or acquire a fully transformed phenotype support the existence of telomerase-independent mechanisms of telomere length maintenance (Bryan *et al.*, 1995, 1997; Blasco *et al.*, 1997). Nevertheless, the consistent repression of telomerase activity in primary cultures and tissues, and its reactivation in the vast majority of human and mouse cancers indicates that the regulation of telomerase is tightly linked to process of immortalization.

Previous studies have established that the telomerase RNA component in mouse (mTR) or humans (hTR) is invariably present in telomerase-positive cells and tissues and that RNA component levels and telomerase activity are coordinately down-regulated in most physiological settings and developing tissues (Feng *et al.*, 1995; Blasco *et al.*, 1995). However, this correlation is not absolute as evidenced by the detection of telomerase RNA in cells and tissues lacking detectable telomerase activity (Feng *et al.*, 1995; Blasco *et al.*, 1995, 1996; Avilion *et al.*, 1996).

Correspondence: GB Morin and RA DePinho
[4]Current address: Department of Pathology, Stanford University School of Medicine, Stanford, CA, 94305, USA
Received 5 February 1998; revised 26 February 1998; accepted 27 February 1998

Such findings have indicated that the regulation of telomerase activity in some cases must involve mechanisms other than those modulating telomerase RNA gene expression. The human telomerase reverse transcriptase protein (previously hTRT, now known as hTERT) has recently been cloned by Nakamura *et al.* (1997) and by others under different names (hEST2, hTLP2, hTCS1, hTRT) (Meyerson *et al.*, 1997; Harrington *et al.*, 1997; Kilian *et al.*, 1997; Nakayama *et al.*, 1998). These studies, using primary and immortalized human cell lines, have established that an additional level of telomerase activity regulation is achieved through mechanisms governing expression of hTERT (Lingner *et al.*, 1997; Nakamura *et al.*, 1997, 1998; Meyerson *et al.*, 1997; Harrington *et al.*, 1997; Kilian *et al.*, 1997). In this study, we have isolated and characterized the mouse ortholog of hTERT with the goals of understanding the regulation of telomerase activity in normal and neoplastic processes in the mouse, providing insights into the immortalization behavior of mouse and human cells, and characterizing the catalytic activities of the mouse protein.

Results and discussion

Isolation and structural characterization of the mouse TERT cDNA

A cDNA fragment of the recently cloned human *TERT*, containing the telomerase motif T and most of the conserved RT motifs (Nakamura *et al.*, 1997), was employed under low-stringency hybridization conditions to screen a mouse embryonal stem cell cDNA library. Nucleotide sequence analysis of a recombinant phage clone identified an open reading frame (ORF) possessing extensive sequence homology with hTERT. This mouse cDNA clone corresponded to the 5′ half of the human *TERT* ORF, the isolation of the remaining 3′ portion of the mTERT ORF was accomplished through a combination of (i) 3′ Rapid Amplification of cDNA Ends–Polymerase Chain Reaction (RACE–PCR), (ii) hybrid Reverse Transcriptase–Polymerase Chain Reaction (RT–PCR) employing mTERT primers directed to the 3′ end of the above cDNA and hTERT primers positioned at or near the end of the human ORF, and (iii) low-stringency hybridization employing a hTERT 3′ ORF probe to screen plasmid subclones derived from an *mTERT*-containing bacterial artificial chromosome (Materials and methods). Assembly of the cDNA clones and PCR products yielded a cDNA fragment spanning 3497 bp and possessing an ATG-initiated ORF of 1122 amino acids predicted to encode a 127 kDa polypeptide with a pI of 10.4 (Figure 1a). Although an in-frame termination codon was not present in the available sequence upstream of the putative initiator codon, several features support the designation of the ATG codon as the initiator including its favorable Kozak consensus context, conservation in mouse and man, reduced conservation upstream of the ATG, and ability to give rise to an *in vitro* translation product similar in size to that produced by the human TERT ORF (see Figure 3). That this predicted mTERT protein indeed represents the mouse telomerase reverse transcriptase catalytic protein is supported by the overall shared amino acid identity (similarity) of 64% (69%) with the human protein (Figure 1a). Moreover,

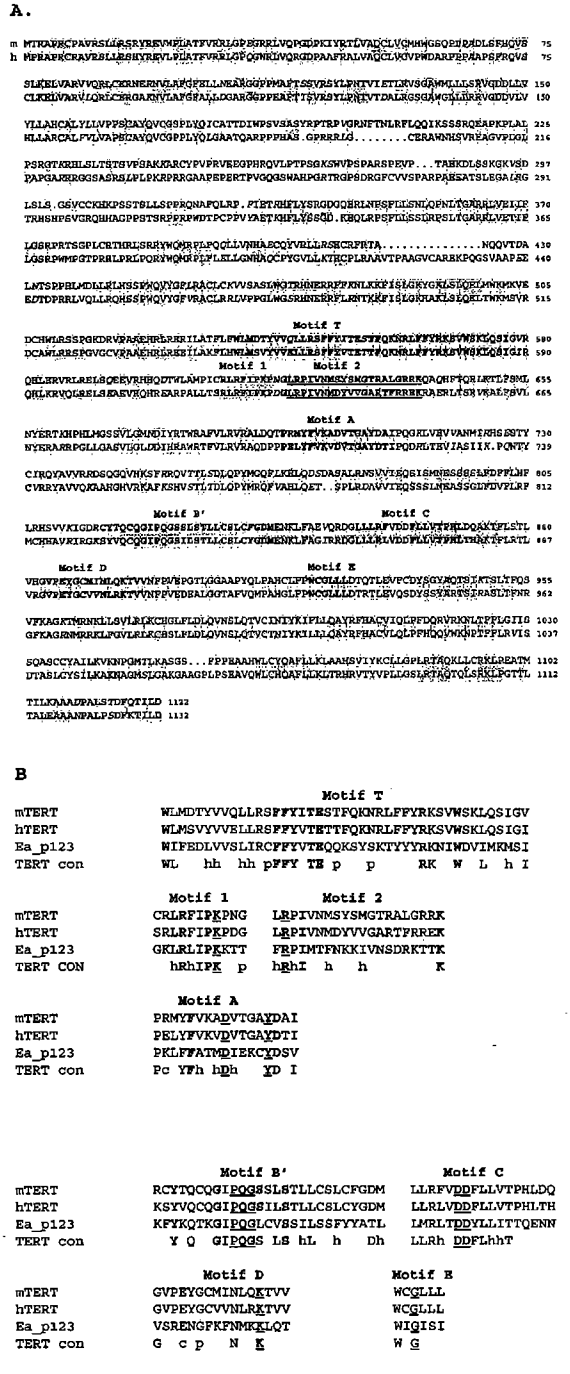

Figure 1 Sequence conservation between mTERT and other telomerase reverse transcriptases. (**a**) Alignment of the predicted amino acid sequences of mTERT (top) and hTERT (bottom). Identical residues between mouse and human are highlighted. Residues which are similar are italicized. Reverse transcriptase motifs and the T motif are shown in bold. Motif 2 (underlined) is contiguous with motif 1. (**b**) Motif alignments for mouse, human and *Euplotes aediculatus*. The consensus sequence for all five known TERTs including the three shown and from *Saccharomyces cerevisiae* (Lingner *et al.*, 1997) and *Schizosacchromyces pombe* (Nakamura *et al.*, 1997) is indicated at the bottom of each motif. Conserved residues are marked according to: bold upper case = 5/5; upper case = 4/5; lower case h = A, L, I, V, P, F, W, M (hydrophobic); lower case c = D, E, H, K, R (charged); lower case p = G, S, T, Y, C, N, Q (polar); underlined = residues conserved in RT motifs

the presence of motif T and all RT motifs from mammals to yeast implies that the catalytic properties of the mTERT protein are conserved over a large phylogenetic distance (Figure 1b).

Chromosomal localization of mTERT

To ascertain the chromosomal location of the *mTERT* gene, we analysed the segregation of an *mTERT*-specific *Eco*RI restriction fragment length polymorphism in The Jackson Laboratory BSS interspecific backcross (Rowe *et al.*, 1994). The allele distribution pattern of the *TERT* locus showed that it cosegregated with *D13Mit8* (Figure 2) (Rowe *et al.*, 1994; Xu *et al.*, 1996). Our analysis indicates that *TERT* fits the composite mouse chromosome 13 map near Mouse Genome Database offset 40, in proximity to the *Srd5al*, *Adcy2*, *Datl* and *Slc9a3* genes. The region to which *mTERT* maps is syntenic to a conserved linkage group near the terminus of the short arm of human Chromosome 5 (band 15). The human gene was shown to map to 5p15.33 (Geron unpublished, accession #AF015950, Meyerson *et al.*, 1997) which also contains the human *Srd5a1*, *Adcy2*, *Dat1* and *Slc9a3* genes. It is notable that, unlike the human gene, the *mTERT* gene does not map to the chromosomal terminus.

Reconstitution of telomerase activity with mTERT protein and human telomerase RNA components

To test directly whether the assembled mTERT cDNA encoded catalytic activity, *in vitro* telomerase reconstitution assays were performed according to Weinrich *et al.* (1997). mTERT was expressed alone or in combination with hTR and telomerase activity was measured by a modified version of the TRAP assay (Weinrich *et al.*, 1997); for a positive control, parallel assays were performed with hTERT and hTR. As shown in Figure 3, the characteristic 6 bp ladder was generated by mTERT in the presence of hTR but not in its absence. TRAP activity was not seen with the mTERT mutant, mTERT-T, which is a truncated version of the polypeptide containing the first 738 amino acids of mTERT, but lacking motifs B', C, D and E. These *in vitro* reconstitution studies demonstrate that the *mTERT* cDNA encodes telomerase RNA-dependent catalytic activity. Furthermore, mTERT and hTR can form a functional ribonucleoprotein complex despite species differences between the telomerase RNAs, including a longer template domain for hTR (Feng *et al.*, 1995; Blasco *et al.*, 1995).

Temporal and spatial patterns of mTERT expression in normal developing tissues

Analysis of human fetal and adult tissues has revealed that telomerase activity closely matches hTERT, but

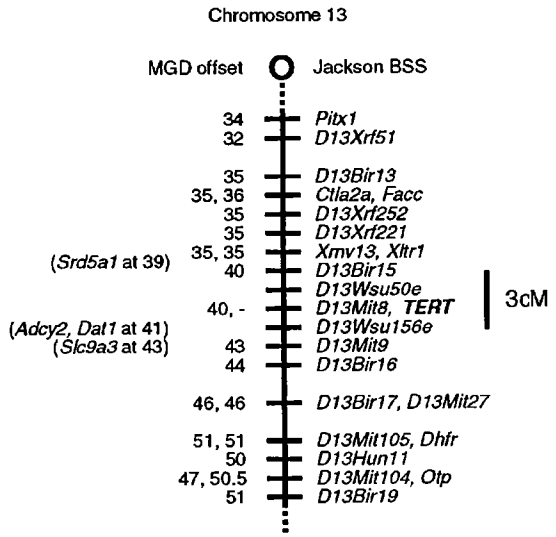

Figure 2 Chromosomal localization of *mTERT*. Depiction of mouse chromosome 13 showing linkage of *TERT* to *D13Mit8* previously mapped in the Jackson Laboratory BSS interspecific backcross. The data give the map order; proximal-*Ctla2a*, *Facc*-4.25 cM ± 2.08 SE-*D13Bir15*-2.13 ± 1.49-*D13Mit8*, *TERT*-2.13 ± 1.49-*D13Mit9*-distal. *TERT* fits the composite mouse chromosome 13 map near MGD offset 40 (cM)

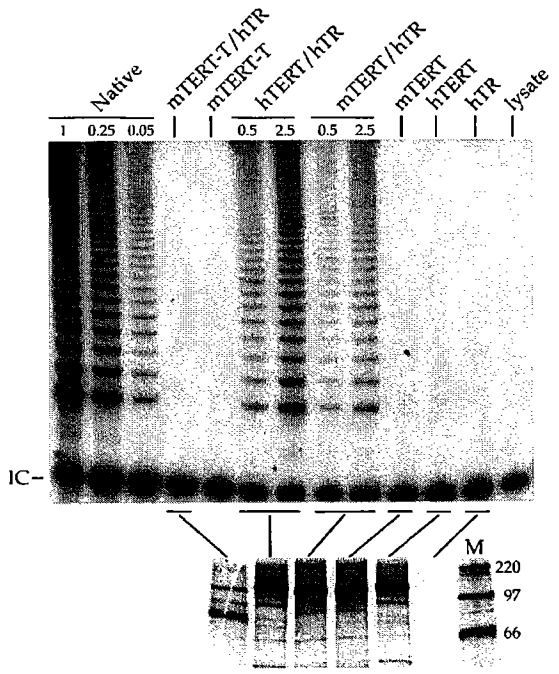

Figure 3 *In vitro* reconstitution of telomerase activity. Upper panel: The indicated combinations of mTERT, hTERT, hTR and control proteins were expressed in a rabbit reticulocyte transcription/translation system (TNT). Then 2.5 and 0.5 μL of the TNT reaction were assayed for telomerase activity using a modified TRAP assay. mTERT-T is a truncated form of mTERT generated by digesting pGRN188 with *Bsp*EI prior to reconstitution, the translated protein contains amino acids 1–738. The lysate lane is the assay of the TNT reaction with no added recombinant DNAs. The native lanes are assays of 1, 0.25 and 0.05 μL of native telomerase from 293 cell lysates. IC = internal control. Lower panel: Documentation of *in vitro* synthesized mTERT proteins. The TNT reactions described above were performed with [^{35}S]methionine; 5 μL of each reaction were analysed by SDS–PAGE to estimate the relative amounts of protein synthesized. The mTERT-T protein has an unexpected molecular weight of 85 kD; the relatively lower signal intensity of the truncated protein reflects that it has 70% of the methionine residues of full length mTERT. The minor amount of full length mTERT in the mTERT-T lane results from incomplete digestion of the parent DNA plasmid. The markers are ^{14}C protein standards (Amersham)

not hTR, expression profiles (Nakamura *et al.*, 1997; Meyerson *et al.*, 1997). To determine whether similar correlations exist in the mouse, RNase protection assays (RPA) were performed on total RNAs derived from staged whole embryos and a panel of postnatal mouse tissues. The specificity of the mTERT probe was confirmed by its detection of a single genomic band on Southern blots and a unique transcript in total RNA derived from telomerase-positive transformed cells on Northern blots (data not shown). It is important to point out that, in all of the studies described in this report, mTERT transcripts are in very low abundance as evidenced by use of an internal β-actin riboprobe control with 500-fold less specific activity than the mTERT riboprobe. As shown in Figure 4, relative to the β-actin internal control, mTERT transcripts were found to be higher in midgestational embryos (stages E9.5 through E15.5) compared with most postnatal tissues. mTERT transcripts were broadly distributed at varying levels in the panel of postnatal tissues assayed. Specifically, mTERT was expressed most prominently in (i) adult organs with detectable telomerase activity (thymus) (Feng *et al.*, 1995), (ii) high renewal and proliferative profiles (intestine and testes), or (iii) significant regenerative capacity (liver). The lung which continues to develop postnatally also showed moderate levels of mTERT expression. Conversely, organs with low proliferative indices (adult brain, heart, muscle and kidney) displayed lower levels of mTERT mRNA. If telomerase reactivation represents an important event in immortalization and tumorigenesis, then the broader expression and less stringent regulation of the mouse TERT gene and telomerase activity may provide a basis for the high rate of cancer in the mouse and explain why mouse cells spontaneously immortalize, whereas human cells do not (Miller, 1991).

Reports of telomerase activity in approximately 90% of mouse tumors indicated that components of the telomerase enzyme may be expressed at high levels in mouse cancers or tumor-derivatived cell lines (Autexier and Greider, 1996). To examine the role of mTERT expression in tumor-associated telomerase activation, RNase protection assays were performed with total RNAs derived from 8 different primary tumors or tumor cell lines including melanoma, lymphoid malignancies, and fibrosarcomas. In all samples, mTERT transcript levels were found to be 5–10-fold higher than in normal mouse tissue or primary cells (data not shown). In contrast, hTERT levels are nearly undetectable in normal human cells, but are present in human tumor cell lines at levels at least 100–fold higher (Nakamura *et al.*, 1997; Meyerson *et al.*, 1997).

Down-regulation of mTERT expression and telomerase activity in terminally differentiated erythroleukemia cultures

The complex pattern of mTERT expression in normal tissues, coupled with the cell type heterogeneity of whole organs, prompted us to examine mTERT and mTR expression and telomerase activity in a well-defined cell cultured-based model for differentiation, the mouse erythroleukemia (MEL) system. Total cellular RNA was extracted from MEL cells cultured for various times in 5 mM hexamethylene bisacetamide (HMBA, a differentiation inducer) and mTERT expression was analysed by RPA. Hybridization signals were quantified and the relative steady state levels of mTERT, normalized to the levels of β-actin, were plotted as a function of time of exposure to the differentiation inducer (Figure 5a and d). Induction of MEL cell differentiation was associated with a biphasic change in the level of mTERT transcripts, a pattern that is also characteristic for Myc mRNA (Rao *et al.*, 1996). Specifically, the mTERT mRNA levels decreased at 2 h, became strongly upregulated between 12 and 36 h, and declined to nearly undetectable levels by 72 h. This late decline in mTERT mRNA levels parallels a marked decrease in telomerase activity (Figure 5c) and coincides with expression of the MEL differentiated phenotype and terminal cell divisions (Lachman and Skoultchi, 1984). The transient discordance between mTERT transcript levels and telomerase activity at early time points may reflect differences in mTERT mRNA and protein stability as human telomerase activity has been estimated to have a

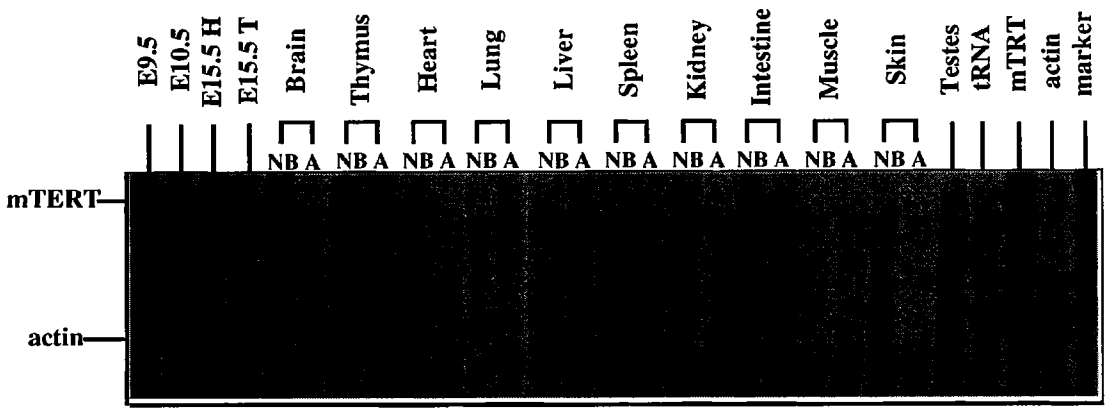

Figure 4 Analysis of the expression pattern of mTERT in normal embryonic, newborn and adult mouse tissues by RNase protection assays. Mosue β-actin was used as an internal control in each reaction, at 1 : 500 lower specific activity than of the mTERT probe. E15.5H = 15.5 day old embryo head portion; E15.5T = 15.5 day old embryo trunk portion, NB = newborn, A = adult

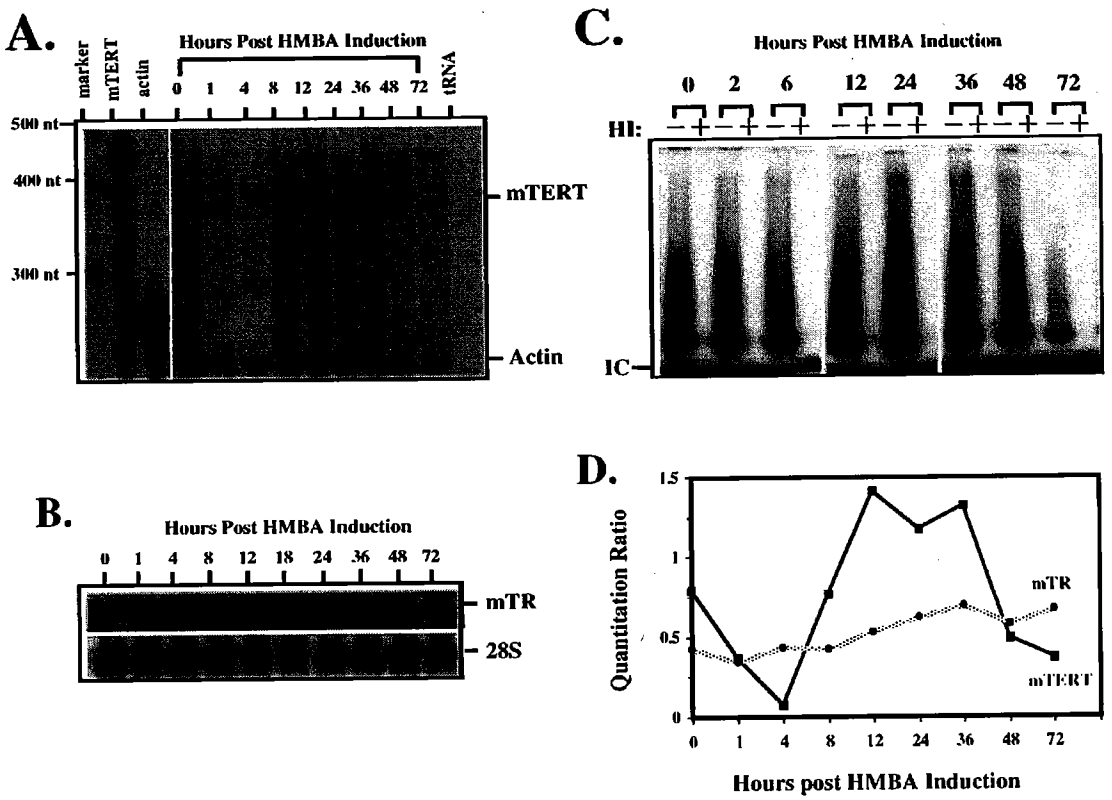

Figure 5 Regulation of mTERT and mTR expressions during MEL differentiation. (**a**) RNase protection assays for mTERT expression were performed as described in Figure 3. Note that the β-actin probe was synthesized at 1 : 500 lower specific activity. (**b**) Northern blot analysis for mTR expression using the same total RNA preparations as in **a**. Hybridization with an oligonucleotide to 28S ribosomal RNA was performed as the loading control. (**c**) Telomerase activity for the corresponding time points, as assayed with the TRAP assay. HI = heat inactivation; IC = internal control. (**d**) Graph of mTERT expression levels determined as described in Figure 3. Solid line = mTERT; dotted line = mTR

half life of approximately 24 h (Holt *et al.*, 1996). In contrast to the highly regulated pattern of mTERT expression, mTR levels remained nearly constant (Figure 5b). Our results in differentiated MEL cultures parallel those reported previously in human HL60 promyelocytic leukemia cells programmed to differentiate to mature granulocytes by treatment with retinoic acid (Meyerson *et al.*, 1997). These findings, together with the tissue studies above, supports the view that the regulation of mTERT gene expression is a key determinant governing telomerase activity during cellular differentiation.

Induction of mTERT in mitogen-induced splenocytes

Lymphocyte activation upon antigen receptor stimulation or mitogen exposure leads to a strong induction of telomerase activity (Buchkovich and Greider, 1996; Bodnar *et al.*, 1996; Morrison *et al.*, 1996). To understand the basis for telomerase activation, mTERT and mTR expression were assayed in adult primary splenocytes cultured in the presence or absence of the T cell-specific mitogens phorbol myristic acid and ionomycin (PMA/I). As shown in Figure 6, mTERT expression levels increased 7–8-fold following PMA/I-treatment compared with 2–3-fold increase in the untreated cultures (Figure 6a and b). In contrast, mTR transcripts were readily detected in primary cultures and remained elevated during the 48 h culture period (Figure 6c). Telomerase activity was detected only in the stimulated cultures and activity increased from 24–48 h (Figure 6d). Similar results were obtained in splenocyte cultures activated with lipopolysaccharide, a potent B cell mitogen (data not shown). These results indicate that mitogen-induced telomerase activation may result from a sharp increase in mTERT gene expression in the face of constant mTR levels. Furthermore, the 2–3-fold increase in mTERT levels in the unstimulated telomerase-negative cultures is consistent with the hypothesis that a threshold level of mTERT may be required for detectable telomerase activity and/or that additional levels of regulation beyond those controlling steady-state mTERT mRNA levels contribute to telomerase regulation in primary lymphocytes.

mTERT and mTR expression in primary, senescent and immortal MEF cultures

Previous studies have shown that dramatic induction of mTR expression correlates with telomerase reactivation following the immortalization of adult mouse fibroblast cultures (Prowse and Greider, 1995; Blasco *et al.*, 1995). As primary mouse embryonic fibroblasts (MEFs) exhibit a high rate of spontaneous immortalization in culture, we examined telomerase activity and mTERT gene expression in early passage, senescent, and post-crisis

MEFs cultures. Early passage MEFs were found to be telomerase-positive and expressed readily detectable mTERT transcript levels (Figure 7a). With continued passage, the entry of cultures into a slow growth senescent phase (passage 10–20) was associated with a decrease in telomerase activity and constant mTERT expression. As cells emerged from crisis and generated immortal cultures, telomerase activity was markedly up-regulated and mTERT expression remained constant (Figure 7a). These findings are in sharp contrast to the highly regulated pattern of mTR gene expression described previously for mouse fibroblasts, showing low mTR levels pre-crisis and dramatic up-regulation in immortal cultures (Blasco *et al.*, 1995).

Previous studies in immortal NIH3T3 fibroblast cultures have demonstrated that telomerase activity is down-regulated as cultures growth arrest via serum starvation or contact inhibition (Xu *et al.*, 1996). To determine whether alteration in mTR or mTERT expression could account for telomerase regulation in these settings, NIH3T3 cells were grown as subconfluent cultures or allowed to achieve confluence for 4 days. Under these conditions, despite a significant decline in telomerase activity (Figure 7d), both mTR and mTERT transcripts remained constant (Figure 7b). These results implicate mechanisms other than the control of steady state mTERT mRNA levels for the decline in telomerase activity in immortalized mouse fibroblasts. *In vitro* evidence indicates that protein phosphatase 2A is a potent inhibitor of telomerase, it will be interesting to determine if post-translational mechanisms such as phosphorylation state are involved in regulating telomerase activity in immortalized mouse fibroblasts (Li *et al.*, 1997).

A most interesting observation to emerge from our studies is the striking contrast between humans and mice in the regulation of TERT gene expression in senescent cultures and in postnatal tissues. Expression of the hTERT gene and concomitant activation of telomerase has been demonstrated to be sufficient for extension of cellular replicative lifespan and bypass of the Hayflick limit for three different primary human cell lines (Bodnar *et al.*, 1998). If the regulation of TERT expression indeed represents a key gatekeeper for the transition to an immortal phenotype in the mouse, as it appears to be for human cells, then the continued expression of TERT in mouse cells and tissues could be an important permissive factor enabling their cancer-prone phenotype. With the

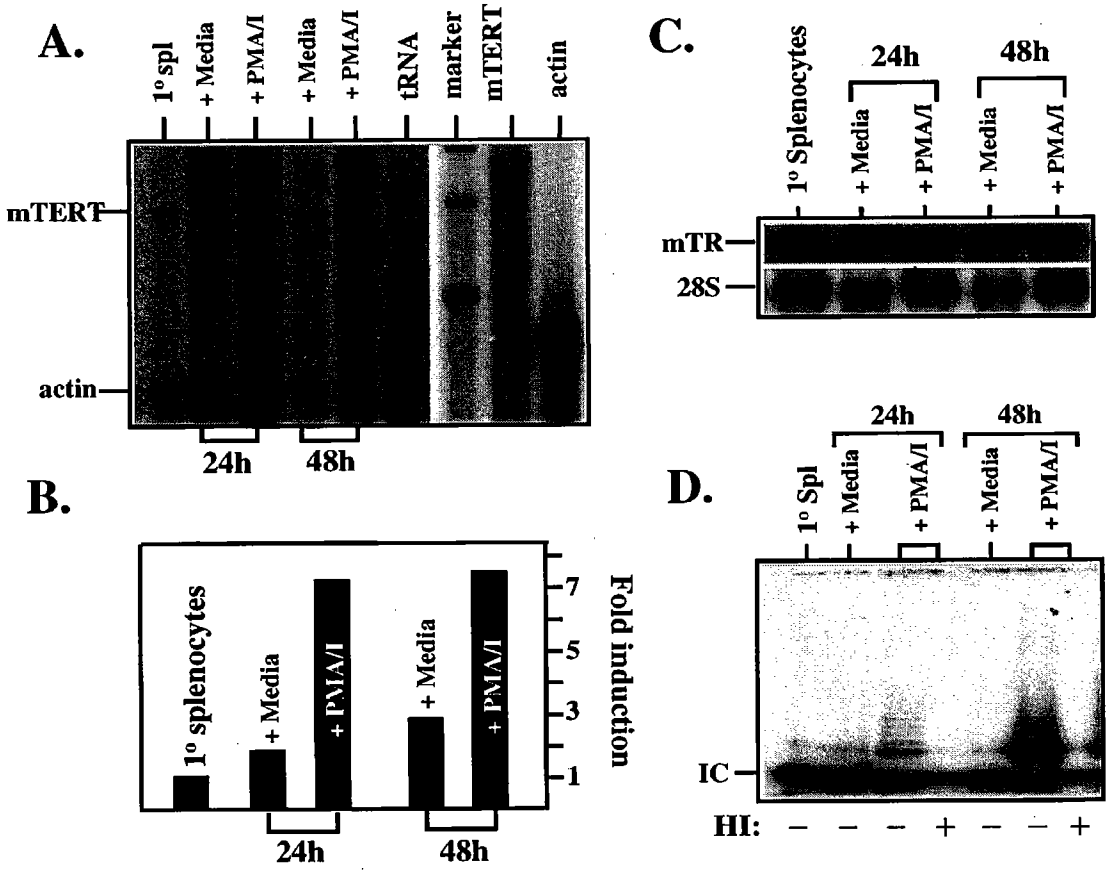

Figure 6 Regulation of mTERT and mTR expression in mitogen-induced lymphocyte proliferation. (**a**) RNase protection assays for mTERT expression as described in Figure 4 after 24 h and 48 h stimulation with PMA/I. 1° spl = primary adult splenocyte. Note that β-actin probe was synthesized at 1 : 500 lower specific activity. (**b**) Histogram representation of mTERT mRNA levels. Fold of induction was calculated by taking level of mTERT expression in 1° unstimulated adult splenocyte as 1.0. (**c**) Northern blot analysis for mTR expression using the same total RNA preparations as in **a**. Hybridization to 28S ribosomal RNA was performed as the loading control. (**d**) Telomerase activity for the corresponding time points, as assayed by the TRAP assay. HI = heat inactivation; IC = internal control

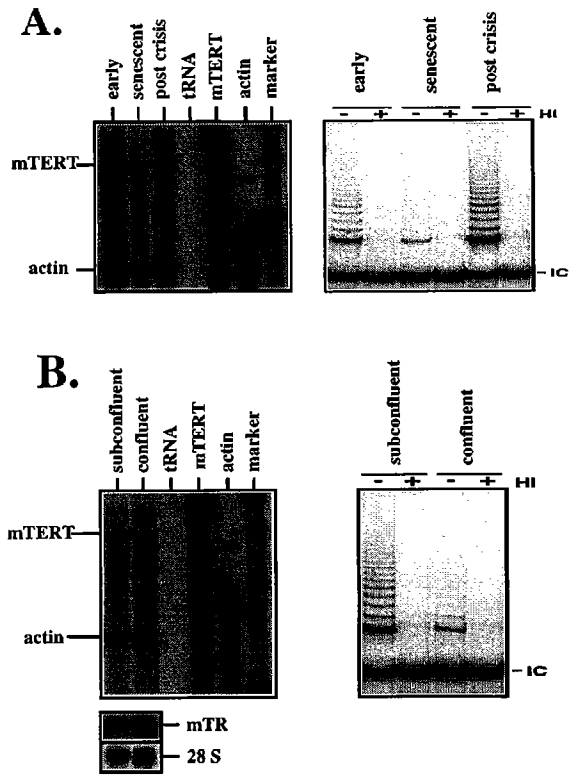

Figure 7 Expression of mTERT in primary embryo fibroblasts (MEF) and NIH3T3 fibroblasts. (**a**) Left panel: mTERT transcript levels were compared among early passage MEFs (passage 4), senescent MEFs (passage 12) and post-crisis MEFs by RNase protection assays as described in Figure 4. Right panel: Corresponding telomerase activity for these MEFs was assayed with the TRAP assay. HI = heat inactivation; IC = internal control. (**b**) Left upper panel: mTERT transcript levels were compared between subconfluent and confluent NIH3T3 fibroblasts by RNase protection assays as described in Figure 4. Left lower panel is Northern blot analysis for mTR expression in the same RNA preparations using 28S rRNA oligo hybridization as loading control. Right panel: telomerase activity as assayed with the TRAP assay. HI = heat inactivation; IC = internal control

isolation of the mouse and human TERT genes, the stage is now set for a comparative analysis of the mechanisms governing TERT gene expression in various physiological settings. Such studies will provide a more detailed understanding of the regulation and relevance of telomerase activation in normal and neoplastic processes *in vivo*.

Materials and methods

Cloning

Low stringency hybridizations were performed with DNA fragments hTERT A (pGRN121 nucleotides 729–1932) and B (pGRN121 nucleotides 2278–3421) probes in prehybridization and hybridization solutions containing 35% formamide at 37°C for 12 h and subsequently washed three times for 30 min in 2 × SSC, 0.1% SDS at 56°C (Nakamura *et al*., 1997). A mouse ES cell λgt10 phage cDNA library (Clontech) was screened with radiolabeled hTERT A probe to initially identify an *Eco*RI fragment containing 1–1970 of the mTERT ORF. Subsequent to obtaining this 5′ cDNA clone, PCR was performed using cDNA generated from mouse testis poly(A)$^+$ mRNA (Clontech), as a template to produce RT–PCR clone 1 which spans motifs 1 through D (Nakamura *et al*., 1997). Briefly, nested PCR (70 cycles in total) was performed using two primer sets with 5′ primers based on the mTERT cDNA clone sequence and 3′ primers based on the human TERT sequence just downstream of motif C. RT–PCR clone 2 was generated by performing nested PCR (55 cycles in total) using primers that anneal to the 3′ end of RT–PCR clone 1 and to the 5′ end of a *Pst*I fragment from the BAC clone that spans RT motifs D and E (Nakamura *et al*., 1997). RT–PCR clone 3 was generated by performing nested PCR (65 cycles in total) using two primer sets that anneal to RT–PCR clone 2 and to the human *TERT* sequence within 30 bp of the stop codon. Finally, 3′ RACE was performed using 3′ RACE (GIBCO/BRL) and primers that anneal to the 3′ end of RT–PCR clone 3 to obtain the 3′ terminus of *mTERT* and the 3′ UTR. All PCR was performed using *Taq* polymerase and 5 ng of cDNA generated from mouse testis poly(A)$^+$ mRNA as template.

Construction of full length mTERT ORF cDNA

Error-free RT–PCR products spanning motif 1 to the 3′ UTR were generated using *Pwo* polymerase (Boehringer Mannheim). Briefly, cDNA was made from mouse testis poly(A)$^+$ mRNA (Clontech) using an oligo dT primer. Nested PCR reaction was then performed with *Pwo* polymerase: 94°C/30′, 68°C/3′ for 30 cycles using primers mTERT.10 (CGTCGATACTGGCAGATGCGG) and mTERT.53 (GTGCTGAGGCTACAATGCCCATGT), followed by 30 more cycles using primers mTERT.9 (CTTTTACATCACAGAGAGCAC) and mTERT.52 (CATGTTCATCTAGCGGAAGGAGACA). The PCR product was cloned into pCRII (Invitrogen), and five independent clones were sequenced. The DNA sequence was identical for all five clones and matched the sequence of the RT–PCR and BAC clones described above. A DNA fragment from this RT–PCR product that starts at a unique *Nhe*I restriction site located in the region of the overlap between this RT–PCR product and the 5′ mTERT cDNA clone and extends to a *Hind*III site in pCRII was transferred to the 5′ *mTERT* cDNA clone to construct the full length *mTERT* ORF plasmid termed pGRN188.

Chromosomal mapping

*Eco*RI polymorphisms between C57Bl/6J and *Mus spretus TERT* loci were identified by screening Southern blots of each DNA (2 μg) digested with *Eco*RI with a mTERT probe spanning nucleotides 1586–1970. This polymorphism was scored in all 94 backcross progeny from the cross C57BL/6J x SPRET/Ei)F1 x SPRET/Ei from The Jackson Laboratory. The allele pattern of *TERT* was compared to the approximately 3200 other loci previously mapped in the Jackson BSS cross and linkage was found to loci on chromosome 13 (Figure 2).

Telomerase reconstitution

In vitro telomerase reconstitution assays were performed as described previously (Weinrich *et al*., 1997). Telomerase activity was measured by a modified version of the TRAP assay (Weinrich *et al*., 1997).

Expression studies

Total cellular RNA was isolated by the LiCl/urea method as previously described (Schreiber-Agus *et al*., 1993). RNase protection assays were performed using HybSpeed RPA kit (Ambion) according to the manufacturer protocol using 40 μg of total RNA per reaction point. RNA probes were synthesized with [α-^{32}P]UTP and T3 RNA polymerase using the MAXIscript kit (Ambion). The DNA template

for mTERT probe synthesis was prepared by inserting a *Bam*HI fragment of mTERT 1585–1970 in an antisense orientation downstream of the T3 promoter in Bluescript KS(+) (Stratagene). The resultant *in vitro* transcript is 474 nt long. Internal control β-actin probe as provided by the kit was synthesized at 1:500 lower specific activity. Reaction products were run on a 6% denaturing polyacrylamide gel, dried and exposed to Biomax MS film (Kodak), for 1–3 days. Quantitation was performed with a phosphorimager (Molecular Dynamics). Northern blot analysis using 20 μg of total RNA was performed as described elsewhere (Shreiber-Agus *et al.*, 1993).

Cell culture

MEFs were isolated on day E13.5 as described previously (Blasco *et al.*, 1997), and grown in Dulbecco's Modified Eagle Medium (DMEM) (Gibco/BRL) supplemented with 10% fetal calf serum, 0.29 mg/mL glutamine, 0.03% penicillin and streptomycin and 25 μg/mL gentamycin sulfate. For NIH3T3 fibroblasts, 10% donor calf serum was substituted for fetal calf serum. In the MEL studies, MEL cell line DS19 were grown in log phase for 48 h 10% DME with glutamine and 0.03% penicillin/streptomycin, and then seeded at 2.5×10^5 mL. Differentiation was initiated by the addition of 5 mM HMBA (Lachman and Skoultchi, 1984). Total cellular RNA was isolated at various time points following induction.

Splenocytes from C57Bl/6J mice were grown in RPMI (Gibco/BRL) containing 10% fetal calf serum, 0.29 mg/mL glutamine, 0.03% penicillin/streptomycin, 25 μg/mL gentamycin sulfate and 55 μM β-mercaptoethanol. Spleens were removed from 14 week old C57BL/6J mice and rinsed one time in PBS containing penicillin/streptomycin and gentimycin sulfate. They were then placed in mesh bags sealed at one end. Cells were placed into single cell suspension in RPMI media and then centrifuged for 10 min at 1500 r.p.m. Splenocytes were seeded in 10 cm suspension plates and activated by the addition of 20 μM phorbol myristic acid (PMA) and 1 μM ionomycin. Total cellular RNA from stimulated and unstimulated plates was isolated at 0, 24 and 48 h. Aliquots of 5×10^5 cells were used for assaying telomerase activity using the TRAP procedure (Kim *et al.*, 1994).

Note added in proof

The nucleotide and amino acid sequence data reported for mTERT in this paper will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number AF051911.

Acknowledgements

We would like to thank Arthur Skoultchi for providing MEL cells, Nicole Schreiber-Agus for critical review of the manuscript, and M Lombardi, S Amshey and R Taylor for expert technical assistance. RAG is supported by NIH Training Grant 5T32Gmo7491. RAD is supported by grants (R01HD28317, R01EY09300, R01EY11267) from the National Institute of Health as well as the Irma T Hirschl award to RAD.

References

Autexier C and Greider CW. (1996). *Trends Biochem. Sci.*, **21**, 387–391.
Avilion AA. (1996). *Cancer Res.*, **56**, 645–650.
Blasco MA, Funk W, Villeponteau B and Greider CW. (1995). *Science*, **269**, 1267–1270.
Blasco MA, Rizen M, Greider CW and Hanahan D. (1996). *Nature Genet.*, **12**, 200–204.
Blasco MA, Lee HW, Hande MP, Samper E, Lansdorp PM, DePinho RA and Greider CW. (1997). *Cell*, **91**, 25–34.
Bodnar AG, Kim NW, Effros RB and Chiu CP. (1996). *Exp. Cell Res.*, **228**, 58–64.
Bodnar AG, Ouellette M, Frolkis M, Holt SE, Chiu CP, Morin GB, Harley CB, Shay JW, Lichsteiner S and Wright WE. (1998). *Science*, **279**, 349–352.
Bryan TM, Englezou A, Gupta J, Bacchetti S and Reddel RR. (1995). *EMBO J.*, **14**, 4240–4248.
Bryan TM, Marusic L, Bacchetti S, Namba M and Reddel RR. (1997). *Hum. Mol. Genet.*, **6**, 921–926.
Buchkovich KJ and Greider CW. (1996). *Mol. Biol. Cell*, **7**, 1443–1454.
Counter CM, Avilion AA, LeFeuvre CE, Stewart NG, Greider CW, Harley CB and Bachetti S. (1992). *EMBO J.*, **11**, 1921–1929.
Feng J, Funk WD, Wang SS, Weinrich SL, Avilion AA, Chiu CP, Adams RR, Chang E, Alsopp RC, Yu J, Le S, West MD, Harley CB, Andrews WH, Greider CW and Villiponteau B. (1995). *Science*, **269**, 1236–1241.
Greider CW and Blackburn EH. (1985). *Cell*, **43**, 405–413.
Greider CW. (1996). *Ann. Rev. Biochem.*, **65**, 337–365.
Harley CB, Futcher AB and Greider CW. (1990). *Nature*, **345**, 458–460.
Harrington L, Zhou W, McPhail T, Oulton R, Yeung DS, Mar D, Bass MB and Robinson MD. (1997). *Genes Dev.*, **11**, 3109–3115.
Holt SE, Wright WE and Shay JW. (1996). *Mol. Cell. Biol.*, **16**, 2932–2939.
Kilian A, Bowtell DD, Abud HE, Hime GR, Venter DJ, Keese PK, Duncan EL, Reddel RR and Jefferson RA. (1997). *Hum. Mol. Genet.*, **6**, 2011–2019.
Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, Coviello GM, Wright WE, Weinrich SL and Shay JW. (1994). *Science*, **266**, 2011–2015.
Lachman HM and Skoultchi AI. (1984). *Nature*, **310**, 592–594.
Li H, Zhao LL, Funder JW and Liu JP. (1997). *J. Biol. Chem.*, **272**, 16729–16732.
Lingner J, Hughes TR, Mann M, Lundblad V and Cech TR. (1997). *Science*, **276**, 561–567.
Meyerson M, Counter CM, Eaton EN, Ellisen LW, Steiner P, Caddle SP, Ziaugra L., Beyersbergen RL, Davidoff MJ, Lin Q, Bachetti S and Weinberg RA. (1997). *Cell*, **90**, 785–795.
Miller RA. (1991). *Cancer*, **68**, 2496–2501.
Morrison SJ, Prowse KR, Ho P and Weissman IL. (1996). *Immunity*, **5**, 207–216.
Nakamura, TM, Morin GB, Chapman KB, Weinrich SL, Andrews WH, Lingner J, Harley CB and Cech TR. (1997). *Science*, **277**, 955–959.
Nakayama J, Tahara H, Tahara E, Saito M, Ito K, Nakamura H, Nakanishi T, Tahara E, Ide T and Ishikawa F. (1998). *Nature Genet.*, **18**, 65–68.
Prowse KR and Greider CW. (1995). *Proc. Natl. Acad. Sci. USA*, **92**, 4818–4822.
Rao G, Alland L, Guida P, Schreiber-Agus N, Chen K, Chin L, Rochelle JM, Seldin MF, Skoultchi AI and DePinto RA. (1996). *Oncogene*, **12**, 1165–1172.
Rowe LB, Nadeau JH, Turner R, Frankel WN, Letts VA, Eppig JT, Ko MS, Thursten SJ and Birenmeirer EH. (1994). *Mammalian Genome*, **5**, 253–274.
Schreiber-Agus N, Horner J, Torres R, Chiu FC and DePinho RA. (1993). *Mol. Cell. Biol.*, **13**, 2765–2775.
Singer MS and Gottschling DE. (1994). *Science*, **266**, 404–409.
Weinrich SL, Pruzan R, Ma L, Ouellette M, Tesmer VM, Holt SE, Bodnar AG, Lichsteiner S, Kim NW, Wright WE, Shay JW, Harley CB and Morin GB. (1997). *Nature Genet.*, **17**, 498–502.
Xu HP, Yanak BL, Wigler MH and Gorin MB. (1996). *Mammalian Genome*, **7**, 16–19.
Yu GL, Bradley JD, Attardi LD and Blackburn EH. (1990). *Nature*, **344**, 126–132.

Query 1 = Mouse TRT (SEQ ID NO:2 from US 6767719)
Sbjct 1 = Human TRT (SEQ ID NO:2 from 09/432,503)

Score = 1348 bits (3490), Expect = 0.0, Method: Compositional matrix adjust.
Identities = 715/1146 (62%), Positives = 839/1146 (73%), Gaps = 38/1146 (3%)

```
Query  1    MTRAPRCPAVRSLLRSRYREVWPLATFVRRLGPEGRRLVQPGDPKIYRTLVAQCLVCMHW  60
            M RAPRC AVRSLLRS YREV PLATFVRRLGP+G RLVQ GDP  +R LVAQCLVC+ W
Sbjct  1    MPRAPRCRAVRSLLRSHYREVLPLATFVRRLGPQGWRLVQRGDPAAFRALVAQCLVCVPW  60

Query  61   GSQPPPADLSFHQVSSLKELVARVVQRLCERNERNVLAFGFELLNEARGGPPMAFTSSVR  120
             ++PPPA  SF QVS LKELVARV+QRLCER  +NVLAFGF LL+ ARGGPP AFT+SVR
Sbjct  61   DARPPPAAPSFRQVSCLKELVARVLQRLCERGAKNVLAFGFALLDGARGGPPEAFTTSVR  120

Query  121  SYLPNTVIETLRVSGAWMLLLSRVGDDLLVYLLAHCALYLLVPPSCAYQVCGSPLYQICA  180
            SYLPNTV + LR SGAW LLL RVGDD+LV+LLA CAL++LV PSCAYQVCG PLYQ+ A
Sbjct  121  SYLPNTVTDALRGSGAWGLLLRRVGDDVLVHLLARCALFVLVAPSCAYQVCGPPLYQLGA  180

Query  181  TTDIWPSVSASYRPTRPVGRNFTNLRFLQQIKSSSRQEAPKPLALPSRGTKRHLSLTSTS  240
             T   P   AS  P R +G         ++  + S +EA  PL LP+ G +R     S S
Sbjct  181  ATQARPPPHASG-PRRRLG--------CERAWNHSVREAGVPLGLPAPGARRRGGSASRS  231

Query  241  VPSAKKARCYPVPRVEEGPHRQVLPTPSGKSWVPSP----ARSPEVPTAEKDLSSKGKVS  296
            +P  K+ R    P  E  P  Q      G++  PS       SP  P AE+  S +G +S
Sbjct  232  LPLPKRPRRGAAPEPERTPVGQGSWAHPGRTRGPSDRGFCVVSPARP-AEEATSLEGALS  290

Query  297  DLSLSG-SVCCKHKPSSTSLLSPPRQNAFQLRP-FIETRHFLYSRGDGQERLNPSFLLSN  354
                S  SV  +H     S   PPR        P + ET+HFLYS GD +E+L PSFLLS+
Sbjct  291  GTRHSHPSVGRQHHAGPPSTSRPPRPWDTPCPPVYAETKHFLYSSGD-KEQLRPSFLLSS  349

Query  355  LQPNLTGARRLVEIIFLGSRPRTSGPLCRTHRLSRRYWQMRPLFQQLLVNHAECQYVRLL  414
            L+P+LTGARRLVE IFLGSRP   G   R  RL +RYWQMRPLF +LL NHA+C Y  LL
Sbjct  350  LRPSLTGARRLVETIFLGSRPWMPGTPRRLPRLPQRYWQMRPLFLELLGNHAQCPYGVLL  409

Query  415  RSHCRFRTA--------------NQQVTDALNTSPPHLMDLLRLHSSPWQVYGFLRACL  459
            ++HC  R A                +    +  +T P  L+ LLR HSSPWQVYGF+RACL
Sbjct  410  KTHCPLRAAVTPAAGVCAREKPQGSVAAPEEEDTDPRRLVQLLRQHSSPWQVYGFVRACL  469

Query  460  CKVVSASLWGTRHNERRFFKNLKKFISLGKYGKLSLQELMWKMKVEDCHWLRSSPGKDRV  519
             ++V   LWG+RHNERRF +N KKFISLGK+ KLSLQEL WKM V DC WLR SPG   V
Sbjct  470  RRLVPPGLWGSRHNERRFLRNTKKFISLGKHAKLSLQELTWKMSVRDCAWLRRSPGVGCV  529

Query  520  PAAEHRLRERILATFLFWLMDTYVVQLLRSFFYITESTFQKNRLFFYRKSVWSKLQSIGV  579
            PAAEHRLRE ILA FL WLM  YVV+LLRSFFY+TE+TFQKNRLFFYRKSVWSKLQSIG+
Sbjct  530  PAAEHRLREEILAKFLHWLMSVYVVELLRSFFYVTETTFQKNRLFFYRKSVWSKLQSIGI  589

Query  580  RQHLERVRLRELSQEEVRHHQDTWLAMPICRLRFIPKPNGLRPIVNMSYSMGTRALGRRK  639
            RQHL+RV+LRELS+ EVR H++   A+   RLRFIPKP+GLRPIVNM Y +G R . R K
Sbjct  590  RQHLKRVQLRELSEAEVRQHREARPALLTSRLRFIPKPDGLRPIVNMDYVVGARTFRREK  649

Query  640  QAQHFTQRLKTLFSMLNYERTKHPHLMGSSVLGMNDIYRTWRAFVLRVRALDQTPRMYFV  699
            +A+  T R+K LFS+LNYER + P L+G+SVLG++DI+R WR FVLRVRA D  P +YFV
Sbjct  650  RAERLTSRVKALFSVLNYERARRPGLLGASVLGLDDIHRAWRTFVLRVRAQDPPPELYFV  709

Query  700  KADVTGAYDAIPQGKLVEVVANMIRHSESTYCIRQYAVVRRDSQGQVHKSFRRQVTTLSD  759
            K DVTGAYD IPQ +L EV+A++I+  ++TYC+R+YAVV++ + G V K+F+  V+TL+D
Sbjct  710  KVDVTGAYDTIPQDRLTEVIASIIK-PQNTYCVRRYAVVQKAAHGHVRKAFKSHVSTLTD  768

Query  760  LQPYMGQFLKHLQDSDASALRNSVVIEQSISMNESSSSLFDFFLHFLRHSVVKIGDRCYT  819
            LQPYM QF+ HLQ++  S LR++VVIEQS S+NE+SS LFD FL F+ H  V+I  + Y
Sbjct  769  LQPYMRQFVAHLQET--SPLRDAVVIEQSSSLNEASSGLFDVFLRFMCHHAVRIRGKSYV  826
```

```
Query  820   QCQGIPQGSSLSTLLCSLCFGDMENKLFAEVQRDGLLLRFVDDFLLVTPHLDQAKTFLST  879
             QCQGIPQGS LSTLLCSLC+GDMENKLFA ++RDGLLLR VDDFLLVTPHL  AKTFL T
Sbjct  827   QCQGIPQGSILSTLLCSLCYGDMENKLFAGIRRDGLLLRLVDDFLLVTPHLTHAKTFLRT  886

Query  880   LVHGVPEYGCMINLQKTVVNFPVEPGTLGGAAPYQLPAHCLFPWCGLLLDTQTLEVFCDY  939
             LV GVPEYGC++NL+KTVVNFPVE   LGG A  Q+PAH LFPWCGLLLDT+TLEV  DY
Sbjct  887   LVRGVPEYGCVVNLRKTVVNFPVEDEALGGTAFVQMPAHGLFPWCGLLLDTRTLEVQSDY  946

Query  940   SGYAQTSIKTSLTFQSVFKAGKTMRNKLLSVLRLKCHGLFLDLQVNSLQTVCINIYKIFL  999
             S YA+TSI+ SLTF   FKAG+ MR KL  VLRLKCH LFLDLQVNSLQTVC NIYKI L
Sbjct  947   SSYARTSIRASLTFNRGFKAGRNMRRKLFGVLRLKCHSLFLDLQVNSLQTVCTNIYKILL  1006

Query  1000  LQAYRFHACVIQLPFDQRVRKNLTFFLGIISSQASCCYAILKVKNPGMTLKASGS---FP  1056
             LQAYRFHACV+QLPF Q+V KN TFFL +IS  AS CY+ILK KN GM+L A G+    P
Sbjct  1007  LQAYRFHACVLQLPFHQQVWKNPTFFLRVISDTASLCYSILKAKNAGMSLGAKGAAGPLP  1066

Query  1057  PEAAHWLCYQAFLLKLAAHSVIYKCLLGPLRTAQKLLCRKLPEATMTILKAAADPALSTD  1116
              EA  WLC+QAFLLKL  H V Y  LLG LRTAQ  L RKLP  T+T L+AAA+PAL +D
Sbjct  1067  SEAVQWLCHQAFLLKLTRHRVTYVPLLGSLRTAQTQLSRKLPGTTLTALEAAANPALPSD  1126

Query  1117  FQTILD  1122
             F+TILD
Sbjct  1127  FKTILD  1132
```